Saskatchewan

A D F

Agriculture Development Fund

FINAL REPORT

DNA MARKER-AIDED SELECTION FOR RESISTANCE TO TAN SPOT AND SEPTORIA NODORUM BLOTCH IN COMMON WHEAT

19970265

Funded by
The Agriculture Development Fund

March 2001
Prepared by: University of Saskatchewan

SAF
Saskatchewan Agriculture & Food

Adding value to agriculture...through research and development

102-00166/

DNA MARKER-AIDED SELECTION FOR RESISTANCE TO TAN SPOT AND SEPTORIA NODORUM BLOTCH IN COMMON WHEAT

Project No. 97000265-96BR

FINAL REPORT

to

SASKATCHEWAN AGRICULTURE DEVELOPMENT FUND

by

G.R. HUGHES, Principal Investigator
Department of Plant Sciences
University of Saskatchewan
51 Campus Drive
Saskatoon SK S7N 5A8

March, 2001

ABSTRACT

The objective of this project was to identify molecular markers which could be used when breeding for resistance to two important components of the leaf spot complex of wheat, tan spot and septoria nodorum blotch. Use of RAPD and PCR primers developed from a RFLP probe linked to resistance to identify markers for tan spot resistance was unsuccessful. The AFLP procedure was used to find markers for septoria nodorum blotch resistance and identified two markers linked to susceptibility with 10 to 12% recombination. Further disease testing to verify the reaction of the lines studied is needed before the real value of these putative markers can be determined. Both studies indicated that RAPD was not suitable for identifying markers in a polyploid species such as wheat.

EXECUTIVE SUMMARY

Septoria nodorum blotch, caused by *Stagonospora nodorum*, and tan spot, caused by *Pyrenophora tritici-repentis*, are two important components of the leaf spot complex which is a widespread disease of wheat in Saskatchewan and should be regarded as the most important endemic disease of wheat in the province. This disease complex causes loss in both grain yield and quality. Yield losses from this complex have been estimated at 10-15% annually in the central and north-eastern cropping regions pf the province.

Control methods for this disease complex include crop rotation, fungicide application and resistant varieties. However, since the level of resistance in most current varieties is low, increased emphasis on breeding for resistance is needed. Host resistance to both diseases is available, is under relatively simple genetic control and can be evaluated at either the seedling or the adult plant stage. Expression of resistance can be influenced markedly by environmental conditions and artificial inoculation is often necessary to ensure effective disease testing, particularly if resistance to a particular disease is being sought. Marker-assisted selection using molecular markers is an efficient way to remove the confusing effects due to the environment and eliminate the need for disease testing, thus speeding up the process.

Therefore, this project was undertaken to find molecular markers which could be used in marker-assisted selection for resistance to septoria nodorum blotch and tan spot and thereby increase the effectiveness of disease resistance breeding and rate of development of leaf spot resistant wheat varieties.

Bulk segregant analysis was used to find RAPD markers for resistance to the necrotic component of the the tan spot syndrome caused by races 1 and 2 of *P. tritici-repentis* in recombinant inbred line populations of crosses involving resistant lines 86ISWMN 2137 and Red Chief, both of which

possess the resistance gene *tsn1*, and susceptible cultivars Glenlea and Kenyon. Almost 600 RAPD primers were screened without identifying a primer which produced a repeatable polymorphism linked to the resistance gene locus. Because of this failure, two PCR primers developed from a RFLP probe linked to gene *tsn1* were tested on the resistant and susceptible parent lines. These produced a 1.2 kb band in both resistant and susceptible lines and a faint 2 kb band in the susceptible lines. Treatment of the 1.2 kb bands from both types of parents with restriction enzyme BamH1 produced two patterns of fragments of different length which were associated with either the A or D genome of common wheat. Comparison of the base pair sequences of these fragments indicated a high degree of similarity among the different fragments and also among fragments from the resistant and susceptible parents. Two sites polymorphic for a small number of bases were revealed by the sequence data; these polymorphisms were specific to one but not both resistant or susceptible parents tested. Any marker based on these polymorphisms would be specific to a particular cross and would not have general application. Thus, PCR analysis also failed to identify useful markers. The high level of genetic similarity in the chromosome region where the necrosis resistance gene tsn1 was located is considered to be a major reason for the failure of the RAPD analysis.

A similar failure of RAPD analysis to detect useful markers for resistance to septoria nodorum blotch was experienced when bulk segregant analysis of an $F_{2:7}$ population of cross 86ISWMN 2137 x Kenyon was performed. For this reason, all further studies on this aspect of the project used AFLP instead of RAPD analysis. Only two primer pairs produced repeatable polymorphisms which initially appeared to be associated with resistance, but subsequent testing did not confirm this association. As well, difficulties with repeatability of results between researchers were encountered. The reasons for such a low degree of polymorphisms and the poor repeatability are not known and when genetic data suggested that resistance in 86ISWMN 2137 was likely controlled by three genes, it was judged that this population was not a suitable one to study.

An $F_{2:8}$ population of cross Red Chief x Kenyon was used in all further studies. Since this cross was more genetically diverse than the previous one and resistance to septoria nodorum blotch in Red Chief is controlled by a single gene, the chances of success were considered to be greater. Bulk segregant analysis indicated that most of the 112 primer pairs tested on this cross produced polymorphisms and that 11 produced repeatable polymorphisms associated with either resistance or susceptibility. When tested on individual $F_{2:8}$ lines, most were only loosely linked with resistance, but three primer combinations were found to be linked with < 15% recombination. However, the data from this series of tests suggested that the disease reactions assigned to some of the $F_{2:8}$ lines

might be incorrect and when data from only those lines whose disease phenotype was considered correct, primer pair E-ACG/M-CTG produced two polymorphisms linked to susceptibility with a 10% recombination. These polymorphisms have potential to be useful markers, but final judgement cannot be made until progeny testing of the individual lines to confirm their disease reaction is completed.

Of the protocols used in this study, the AFLP procedure proved to be the most powerful as judged by the number of polymorphisms revealed; this confirms results from other laboratories. RAPD was not suitable for this study which involved crosses with limited genetic variability and investigation of a chromosome region of a complex polyploid species and which, in the case of tan spot resistance, was highly conserved. This strongly suggests that only the more powerful techniques such as AFLP or microsatellites should be used in future attempts to identify molecular markers in wheat. This study also stressed the importance of careful testing to correctly determine the disease phenotype of the lines of the mapping population. Suspected incorrect phenotyping proved to be a limitation in correctly assessing the usefulness of putative markers for septoria nodorum resistance, a disease more difficult to test for than tan spot.

This difficulty emphasizes the desirability of having available molecular markers which could be used in resistance breeding programs, and thus additional research to establish the value of the potential markers identified is needed. Further research investigating of the value of microsatellites to find markers for resistance to this disease is desirable. However, since seedling testing for tan spot reaction is, in general, reliable and repeatable, and, given the lack of success of finding markers this study, further research to identify markers for tan spot resistance should not considered a high priority. It is acknowledged that AFLP analysis was not used in the tan spot resistance marker study, and if it had, results may well have been different.

OVERALL OBJECTIVES OF PROJECT

To develop DNA-based molecular markers for use in screening for resistance to the leaf spot diseases tan spot and septoria nodorum blotch in all classes of common wheat so as to improve the effectiveness of disease screening and the efficiency of developing disease resistant cultivars.

DEVELOPMENT OF MAPPING POPULATIONS

Random inbred line (RIL) populations were developed by single seed descent or double haploid production using the wheat-maize procedure for a number of crosses involving parents resistant to septoria nodorum blotch and/or tan spot. The following populations were developed:

Doubled Haploid Populations

(a) *Septoria nodorum blotch resistance* studies

86ISWMN 2137 x Kenyon

EE8(Ag) x Kenyon

CDC Clair x EE8

RIL Populations

(a) *Septoria nodorum blotch and tan spot resistance* studies

EE8 x Kenyon F2:9 (59 lines)

Red Chief x Kenyon F2:8 (59 lines)

86ISWMN 2137 x Glenlea F7

86ISWMN 2137 x Kenyon F7

2095 x Kenyon F2:7 (81 lines)

Coker 76-35 x Kenyon F2:6 (83 lines)

Missouri Queen x Kenyon F2:5 (73 lines)

(b) *Tan spot resistance studies*

CDC Clair*2/Erik

86ISWMN 2137 x Kenyon

(b) *Tan spot resistance studies*

Glenlea x Erik F7

Kenyon x Hadden F7 (49 lines)

In all crosses, Kenyon and Glenlea were the susceptible parents.

DISEASE TESTING OF MAPPING POPULATIONS

Septoria nodorum blotch

An F2:7 RIL population of EE8 x Kenyon (58 lines) and an F2:7 RIL population of Red Chief x Kenyon (58 lines) were tested twice for septoria nodorum blotch reaction at the seedling stage in the growth room. These tests identified resistant and susceptible lines to use in forming bulks for bulked segregant analysis. The testing protocols for disease reaction to septoria nodorum blotch follows.

Disease testing was conducted in a growth room operating at 21°C/18°C day/night temperatures and a 16 h daylength. Plants at the 3-leaf stage were inoculated with the Kelvington isolate of *Stagonospora nodorum* using a pycnidiospore suspension prepared from 8-10 day-old cultures grown on V8-medium under continuous fluorescent light at room temperature. The spore suspension, adjusted to a conccentration of 1 x 10^6 spores per ml was sprayed on the plants until run-off. The plants were then placed in a moist chamber for 48 hours and then returned to the growth room. Plants were rated for disease reaction seven days after inoculation using a 1-5 scale where 1 = resistant and 5 = susceptible.

Segregation data from these crosses suggest that a single major gene in Red Chief and two genes in EE8 control resistance. Modifying genes also appear to be influencing disease reaction. The two-gene control in EE8 was confirmed by testing 70 doubled haploid lines developed from the EE8 x Kenyon cross; these genes appear to act additively.

Tan Spot

$F_{2:7}$ lines of crosses Glenlea X 86ISWN 2137, Kenyon X 86ISWN 2137, and Kenyon X Red Chief were also tested for tan spot reaction at the seedling stage using isolate Ptr 92-164 of *P. tritici-repentis*. This isolate induces the necrotic lesion reaction. The disease testing protocol used was as follows:

Single spore cultures of Ptr 92-164 were grown on V8-PDA medium and inoculum was prepared as described by Lamari and Bernier (1989). Seedlings at the two leaf stage were sprayed until runoff with a conidial suspension (3000 conidia/ml + two drops of Tween 20/100 ml) and incubated for 24 hours under continuous leaf wetness at 20°C and a 16-hour photoperiod. The pots were then transferred to benches in the growth room at 20/17°C (day/night) and 16-hour photoperiod, until rated for disease reaction seven days later using a 1-5 scale where,

1 = Small, dark brown to black spots without any surrounding chlorosis or tan necrosis

2 = Small, dark brown to black spots with very little chlorosis or tan necrosis

3 = Small, dark brown to black spots completely surrounded by distinct chlorotic or tan necrotic ring, lesions are not coalescing

4 = Small, dark brown to black spots completely surrounded with tan or chlorotic necrotic zones some of the lesions coalescing

5 = The dark brown or black centers may or may not be distinguishable most lesions consist of coalescing chlorotic or tan necrotic zones

Segregation data indicated that tan spot resistance in these crosses was controlled by a single recessive gene. Other tests determined that the same resistance gene was present in all three resistant parents and that this gene was the same as *tsn1*, which has been mapped to chromosome 5B.

DEVELOPMENT OF MOLECULAR MARKERS FOR TAN SPOT RESISTANCE

INTRODUCTION

Tan spot, caused by the fungus *Pyrenophora tritici-repentis,* is an important foliar disease of wheat throughout the world and particularly in western Canada. Tan spot not only causes significant grain yield loss but also causes reduction in the quality of grain produced. In recent years, it has been the major component of the leaf spot complex of wheat in western Canada, particularly on durum wheat. Two phenotypically distinct symptoms are associated with this disease, tan necrosis and extensive chlorosis.

Races have been identified in this fungus and differ in their ability to produce the necrotic and chlorotic symptoms characteristic of this disease. Race 1, capable of producing both necrosis and chlorosis, and Race 2, which produces only the necrotic symptoms, are the two predominant races found in Saskatchewan. Resistance to necrosis produced by both these races is controlled by a single recessive gene, *tsn1*. Since the necrotic symptom is the most common symptom to which resistance is required in wheat cultivars grown in Saskatchewan and since resistance to this symptom is controlled by a single gene, identification of a molecular marker(s) that could be used in marker-assisted screening for resistance to necrosis would be of considerable value to western Canadian wheat breeders. Therefore, the objective of this study was to identify such molecular markers.

MATERIALS AND METHODS

Population Development and Disease Screening

Three populations, $F_{2:7}$ lines of crosses Glenlea x 86ISWMN 2137, Kenyon x 86ISWMN 2137, and Kenyon x Red Chief, were studied to identify RAPD markers linked to resistance to the necrosis symptoms of tan spot of wheat. The method of development of these populations was described earlier. Approximately 100 $F_{2:7}$ lines of each cross were tested for reaction to a necrosis-inducing isolate of the tan spot fungus, Ptr 92-164 (race 2), in growth room tests to establish their disease phenotype. A minimum of 16 plants of each line were tested.

Based on these disease tests, 10-15 of the most resistant and the most susceptible lines of each cross were identified and DNA extracted from one plant of each line. Plants from which DNA was

extracted for the molecular studies were grown to maturity and their progeny tested for tan spot reaction to verify the disease reaction phenotype of each parent plant. Selection of the lines used for the bulk segregant analysis was based on the progeny test results.

DNA Extraction

DNA extraction followed the method developed by Procunier et al. (1991). From each $F_{2:7}$ plant, one tiller was harvested at the 3-4-leaf stage for DNA extraction. From this tiller approximately 0.2-0.3 g fresh tissue was cut into small pieces, placed in a 1.5-ml microfuge tube, and frozen by the addition of liquid nitrogen. The frozen tissue was macerated and 0.5 ml 2X CTAB buffer (Appendix III) at 65°C was added to each tube and the contents mixed well. The tubes were then placed in a 65°C water bath for 10 min before adding 0.5 ml chloroform/isoamyl alcohol (24:1) to each tube. The contents of each tube were thoroughly mixed using a vortex mixer and the suspension microfuged at 13,000 RPM for 10 min.

After microfuging, the upper aqueous phase (about 450 µl) was decanted into a new tube and a 0.1 volume (45 µl) of 10% CTAB at 65°C and one volume of 24:1 chloroform/isoamyl alcohol were added to each tube, mixed and microfuged again at 13,000 RPM for 10 min. The supernatant (about 450 µl) was transferred to a new tube and two volumes of cold (-20°C) 95% ethanol added. The tubes were then placed on ice for 5 min before precipitating the DNA by centrifugation (13,000 RPM for 10 min). To purify the DNA, the 95% alcohol was poured off, 500 ml of cold (-20°C) 70% ethanol added to each tube which was centrifuged for 5 min. The alcohol was then decanted, the remaining DNA pellet air-dried at room temperature, resuspended in 100 µl of double distilled water and kept at 4°C overnight. Finally, each DNA sample was quantified on the basis of UV absorption at 260 nm (GeneQuant, Pharmacia LKB Biochrom Ltd), diluted to a final concentration of 25 ng/µl and stored at -20°C until use.

Bulk Segregant Analysis

Two attempts were made to identify RAPD markers linked to tan spot resistance gene using bulk segregant analysis. In each case a resistant and a susceptible bulk was created by bulking DNA from lines which had shown a high level of resistance or susceptible and whose disease reaction had been confirmed by progeny testing.

In the first attempt the bulks were based on five lines from the cross Glenlea X 86ISWMN 2137 and two lines from cross Kenyon X 86ISWMN 2137. By using two different crosses to form the bulks it was hoped to develop cross non-specific RAPD markers.

Resistant bulk composition	Susceptible bulk composition
Line 306 - Glenlea X 86ISWMN 2137	Line 312 - Glenlea X 86ISWMN 2137
Line 304 - Glenlea X 86ISWMN 2137	Line 305 - Glenlea X 86ISWMN 2137
Line 264 - Glenlea X 86ISWMN 2137	Line 291 - Glenlea X 86ISWMN 2137
Line 256 - Glenlea X 86ISWMN 2137	Line 287 - Glenlea X 86ISWMN 2137
Line 229 - Glenlea X 86ISWMN 2137	Line 286 - Glenlea X 86ISWMN 2137
Line 199 - Kenyon X 86ISWMN 2137	Line 176 - Kenyon X 86ISWMN 2137
Line 196 - Kenyon X 86ISWMN 2137	Line 172 - Kenyon X 86ISWMN 2137

In the second attempt lines from the cross Kenyon X Red Chief were used. The resistant and susceptible bulks were each based on ten lines.

Primer Screening and Amplification Conditions

The RAPD primers used in this study were obtained from Operon Technologies Inc. USA, and the Biotechnology Laboratory, University of British Columbia, Canada. The PCR reaction mixture consisted of 1unit of Taq DNA polymerase, 25 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.2 µM of primer and 200 µM each of dTTP, dATP, dCTP and dGTP (Gibco BRL, Canada). The reaction mixture was brought to a volume of 25 µl by addition of sterile double distilled water and overlaid with 25 µl of mineral oil.

The PCR reaction was done using a RoboCycler® Gradient 40 thermocycler (Stratagene®, La Jolla, California, USA). The PCR amplification procedure was an initial denaturation at 94°C for 6 min, followed by 45 cycles of PCR, each cycle involving denaturation at 92°C for 1 minute, annealing at 36°C for 1 min and extension at 72°C for 1 min. After a final extension cycle of 72°C for 6 min, the reaction product was maintained at 6°C until loaded onto a gel for electrophoresis.

Gel Electrophoresis

After PCR amplification, 4 µl of gel loading buffer (6X) was added to the PCR reaction product which was then loaded onto a 1.5 % agrose gel in 1X TAE and electrophoresed at 80 to 100 V for 2 - 3 h. After electrophoresis, the gel was stained with ethidium bromide (10 mg/ml), viewed under a UV transilluminator (Cole-Parmer Instrument Co. Chicago, France) and photographed using the gel documentation system UVP ImageStore 7500 (DiaMed Lab Supplies Mississauga, Ontario).

PCR Primer Development and Amplification Conditions

PCR primers from RFLP probe Xbcd 183 were developed using sequences provided by Dr. J. Faris, Kansas State University. This probe is closely linked to resistance gene (*tsn1*) controlling the necrosis component of tan spot. These PCR primers amplify single strong band of size 1.2 kb, which when subjected to restriction digestion was expected to give polymorphisms (Faris, personal

communication). The PCR primers were designed to have the following sequences and were synthesized by Gibco BRL, Canada:

BCD 183 F: 5'GGATATAACCAGTGTTACTTTCCAACCGG3'

BCD 183 R: 5'CACGGCGCTGCACCTCATCTAATTG3'

PCR using these primers was done with a RoboCycler®, Gradient 40 thermocycler (Stratagene®, La Jolla, California, USA) and an amplification protocol of an initial denaturation cycle of 94°C for 3 min followed by 30 cycles of PCR, each cycle involving 94°C for 1 min, 53°C for 1 min and 72°C for 2 min, with a final extension cycle of 72°C for 7 minutes. After PCR, the reaction product was held at 6°C until used. The PCR reaction mixture consisted of 1unit of Taq DNA Polymerase, 200 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.2 µM each of the forward and backward primer, and 200 µM each of dTTP, dATP, dCTP and dGTP. The reaction was brought to a volume of 50 µl by addition of sterile distilled water and overlaid with 25 µl of mineral oil before the PCR reaction was commenced.

The 1.2 kb band obtained by PCR amplification was digested with the following restriction enzymes.

1) Rsa I	6) Eco RV	11) Hae III	16) Hinf I
2) Mse I	7) Hind III	12) Apa I	17) Mbo I
3) Tha I	8) Xho I	13) Sau 3 AI	18) Tsp 5091
4) Alu I	9) Bgl II	14) Sma I	
5) Taq I	10) Eco RI	15) Msp I	

Digestion was performed in the buffer and conditions best suited for the particular restriction enzyme. The digestion product was then subjected to gel electrophoresis to reveal any resulting polymorphisms.

Cloning and Sequencing of the 1.2 kb Fragment

The 1.2 kb fragment amplified by the PCR primers BCD 183F and BCD 183R was cloned and then sequenced as a first step for possible development of sequence characterized amplified region (SCAR) markers. The 1.2 kb fragment was cloned into the plasmid *bluescript* using the TA cloning kit (Invitrogen BV, San Diego). *Escherichia coli* was then transformed with recombinant plasmids and plated on a selective media containing 50 µg/ml X-gal and 50 µg/ml ampicillin. Colonies that grew were cultured overnight in liquid broth media (Bacto Tryptone-10 g/L, yeast extract-5 g/L, NaCl-10 g/L, 1,000 mld sterile double distilled water) at 37°C on a orbital shaker. Plasmid extraction was done using the Wizard™ Miniprep kit (Promega, Wisconsin, USA). To determine if the correct fragment had been cloned into the plasmid, various bacterial colonies were

picked and the cloned fragments were amplified using primers T_7 and M_{13} designed for amplifying cloned fragments in the plasmid *bluescript*.

Plasmids with the correct sized insert were sequenced by the Plant Biotechnology Institute, Saskatoon. The computer program DNA-MAN was then used to align the sequences so that base-pair comparisons could be made.

Identification of the Fragments Amplified by PCR Primers

Identification of the different fragments amplified by primers BCD 183F and BCD 183R was attempted. Using these primers, DNA from *Triticum aestivum*, *T. durum*, *T. tauschii*, and cytogenetic stocks nullisomic 5A tetrasomic 5B, nullisomic 5B tetrasomic 5A, nullisomic 5B tetrasomic 5D, nullisomic 5D tetrasomic 5A, and nullisomic 5D tetrasomic 5B lines was amplified and digested with restriction enzyme Bam HI. Gel electrophoresis was performed to identify the different fragments produced.

RESULTS

RAPD Analysis - Use of Bulks composed of lines from crosses Glenlea x 86ISWMN 2137 and Kenyon x 86ISWMN 2137

Resistant and susceptible bulks comprised of F2:7 lines from the crosses Glenlea x 86ISWMN 2137 and Kenyon x 86ISWMN 2137 were screened with 440 UBC primers (primers UBC #1-140 and 500-800). Of these primers, 119 produced polymorphisms in the first screening but only 40 primers produced polymorphisms when tested again. However, for most of the 40 primers, the banding pattern produced in the second test was different to that in the first, indicating that the polymorphisms were not repeatable and that these primers were not useful. Two primers, UBC 608 and UBC 618, did produce repeatable polymorphisms when tested on the bulks, but failed to produce reliable and repeatable polymorphisms when tested on the individual lines constituting the bulks. Because of these negative results, work with these populations was discontinued.

RAPD Analysis - Use of Bulks from cross Red Chief x Kenyon

A second attempt to identify RAPD markers for tan spot resistance was made using F7 lines of cross Red Chief (Resistant winter wheat) x Kenyon (Susceptible spring wheat), the most genetically diverse cross in this study. Resistant and susceptible bulks were each created by compositing DNA from 10 lines of the appropriate disease reaction.

A total of 240 primers were screened, 140 Operon primers (series I, H, K, L, M, N and O) and

UBC primers #601-700. Fourteen primers produced polymorphisms in the first and second screening, but only five produced the same banding pattern on the bulks in both tests. These were Operon primers OPI1, OPI18 and OPN1, and UBC 533 and UBC 628 .

When tested on the 20 individual lines used to create the bulks, no linkage between the band created by primers OPI1 and OPI18 and resistance was found. OPN1 produced a band that was linked to resistance with a recombination value of about 25 cM (5 out of the 20 lines screened were recombinants). However, when an additional 20 lines were screened, the linkage disappeared. The marker bands produced by primers UBC 628 and UBC 533 were also associated with resistance but the linkage was loose with recombination values of approximately 45 cM (18 recombinant lines out of 40 screened) and 35 cM (14 recombinant lines out of 40 screened), respectively. None of these markers were linked closely enough to resistance to be of value in marker-assisted selection.

Primers UBC102 and UBC109, which in previous work (Stock, W.S. 1996. M.Sc. thesis, University of Manitoba) produced markers linked to the necrosis resistance gene *tsn1*, failed to produce any polymorphisms when tested on the Red Chief x Kenyon bulks and lines. Because of the lack of positive results, it was decided to cease work with RAPD and look to other ways of finding useful markers.

PCR Primer Analysis

Since both Stock (1996) and Faris et al. (1996) reported that tan spot resistance gene *tsn1* was located on chromosome 5BL, it was decided to investigate the value of PCR primers derived from RFLP probes mapped to chromosome 5BL. Faris et al. (1996) found that RFLP probe Xbcd 1030 was linked to gene *tsn1*, but since this probe detected multiple fragments it was not considered suitable for development of PCR primers. However, Dr. Faris (personal communication) provided primer sequences from a related probe Xbcd 183 which he had found to be tightly linked to *tsn1* at a distance of 0.6 cM in a durum wheat mapping population. Later work indicated that recombination in this durum population was suppressed so that the recombination value of 0.6 cM underestimated the true linkage between Xbcd 183 and *tsn1*. The true linkage was not known.

After optimization of the PCR amplification conditions, PCR primers XBCD 183F and XBCD 183R were tested on resistant cultivars Erik (R1) and Red Chief (R2) and susceptible cultivars Glenlea (S1) and Kenyon (S2). These primers produced one monomorphic 1.2 Kb band in all cultivars and a faint 2 Kb in the susceptible cultivars (Fig. 1). Since Faris (personal communication) had indicated that the 1.2 Kb band was associated with the chromosome region where the resistance

gene was located, only this band was studied further. Although the 2Kb band was polymorphic and reproducible, it was difficult to detect because of the faintness of its expression.

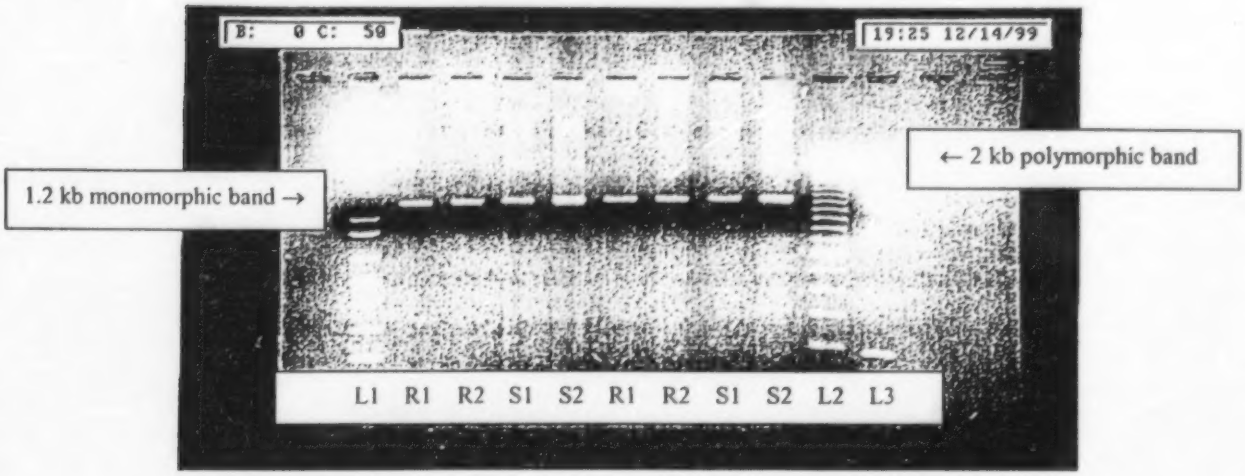

Figure 1. The 1.2 kb band observed in resistant (R1 and R2) and susceptible (S1 and S2) cultivars, and the 2 kb band observed only in susceptible (S1 and S2) cultivars when amplified by PCR primer XBCD 183.

In an attempt to find a polymorphism between the resistant and susceptible cultivars, the 1.2 Kb band was digested with restriction enzymes. While most of the enzymes effectively digested this band, none of the digestion products produced polymorphisms. Digestion with restriction enzyme Bam HI resulted in production of one 1.2 Kb band and two bands, 800 bp and 400 bp in size, respectively. This indicated that the 1.2 KB band produced by the XBCD primers was composed of more than one fragment.

In preparation for sequencing, 1.2 Kb fragments identified from the resistant cultivars were inserted into plasmids and these plasmids were then digested with restriction enzyme Bam HI to identify the correct fragment to be sequenced. Three patterns of fragments were produced: an undigested fragment 1.2 Kb in size (F3), two fragments 800 bp and 400 bp in size (F2) and two fragments 700 bp and 500 bp in size (F1) (Fig. 2). Fragment pattern F1 was found only rarely, indicating that these fragments were amplified at a low frequency during PCR. Plasmids with the 1.2 Kb fragment from the susceptible cultivars produced only two patterns of fragments: an undigested 1.2 Kb fragment and two fragments 800 bp and 400 bp in size (Fig. 2). The occurrence of three

fragment patterns suggested that all copies of chromosome 5 (i.e. 5A, 5B and 5D) were being amplified. Similar results were obtained by Dr. Faris (personal communication).

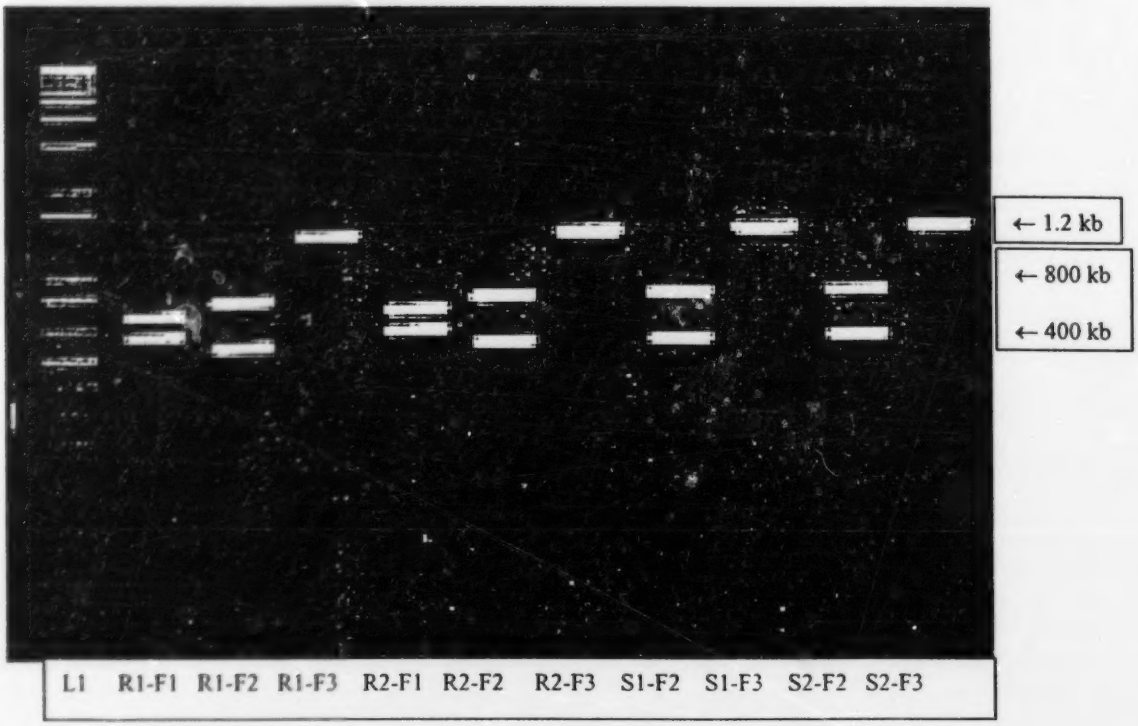

Figure 2. The PCR amplification and restriction digestion by Bam HI of different plasmids containing the 1.2 kb inserted fragment. Three types of fragment patterns, F1, F2 and F3, were obtained from resistant sources (R1-F1, R1-F2, R1-F3, R2-F1, R2-F2, R2-F3) and two types from susceptible sources (S1-F1, S1-F2, S2-F1, S2-F2).

Attempts were then made to associate these fragment patterns with the appropriate genome by amplification of DNA from common wheat, durum wheat, *T. monococcum* and *T. tauschii* lines and aneuploid stocks for chromosome 5and subsequent digestion by restriction enzyme Bam HI. The 1.2 Kb fragment (pattern F3) was produced by *T. tauschii* (genome DD) and aneuploid lines N5B/T5A, N5A/T5D and N5A/T5B, indicating that this fragment associated with the D genome (Fig. 3). The 800 bp and 400 bp fragment pattern (F1) was produced by aneuploid lines N5B/T5A and N5D/T5B, indicating that this fragment pattern was associated with the A genome (Fig. 3). Both the F1 and F3 fragment patterns were produced by the resistant and susceptible common wheat cultivars

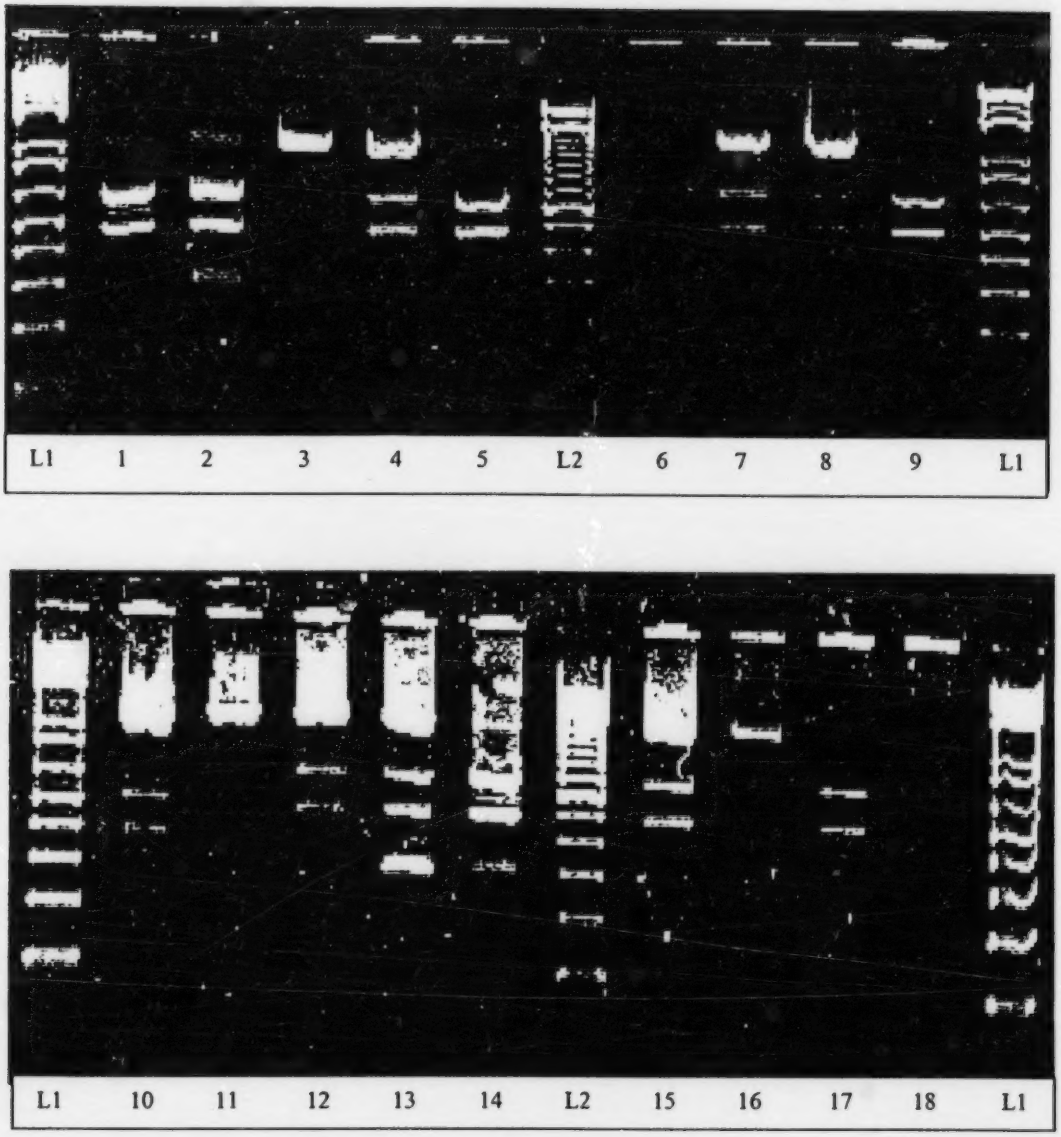

Figure 3. Banding patterns shown by the different genotypes after amplification with the XBCD primers and digestion with restriction enzyme BamH1.

1 = DT 926 (AABB)	2 = TM 23 (AABB)	3 = *T. tauschii* (DD)	4 = Katepwa
5 = DT 925 (AABB)	6 = N5B/T5D (AADD)	7 = Glenlea (AABBDD)	8 = N5B/T5A (AADD)
9 = DT 691 (AABB)	10 = N5A/T5D (BBDD)	11 = N5A/T5B (BBDD)	12 = Kenyon (AABBDD)
13 = 2137 (AABBDD)	14 = DT 522 (AABB)	15 = Red Chief (AABBDD)	16 = Erik (AABBDD)
17 = N5D/T5B (AABB)	18 = N5D/T5A (AABB)	L1 = 1 kb ladder	L2 = 100 bp ladder

(genome AABBDD), but only the 800 bp and 400 bp fragment pattern was produced by the durum wheat lines DT 926, DT 925, DT 961 and DT 522 (genome AABB) and *T. monococcum* line TM 23 (genome AA). This was expected based on the association of the fragment patterns observed on the

aneuploid lines.

Identification of the fragment pattern associated with the B genome was inconclusive. The 1.2 Kb fragment was produced by aneuploid lines N5A/T5D and N5A/T5B, but not by N5D/T5B where the 800 bp and 400 bp fragment pattern was produced. A faint 1.2 Kb band was produced by the durum wheat lines. The 700 bp and 500 bp fragment pattern was not produced by any of the genotypes tested. Tentatively, it is suggested that fragment pattern F2 (800 bp and 400 bp fragments) is associated with the B genome also.

Sequencing of the various fragments showed that there was a high degree of similarity between the three fragment patterns and between the fragments from the resistant and susceptible cultivars. Only a few base pair differences were found between pattern F2, the 800bp and 400 bp fragments, and pattern F1, the 700 bp and 500 bp fragments. In addition, the restriction enzyme Bam HI cutting site was found to be at the same location regardless of which fragment pattern was produced. This finding was surprising since digestion with Bam HI produced two distinctly different fragment patterns, F1 and F2, and provides no explanation for their production.

The sequence data suggested that SCAR markers could be designed to identify the gene for resistance to the necrosis component of tan spot. Two sequences were identified which could be used. One was based on a 1- base pair difference, the other on a 3-base pair difference. However, since these polymorphisms were present in cultivars Erik and Kenyon, but absent in Red Chief and Glenlea, the markers that could be developed very likely would be cross-specific and thus of limited value. For this reason, and since there was no assurance that effective SCAR markers could be designed within the time remaining for the project, this aspect of the project was terminated.

DISCUSSION

The objective of this portion of the study, to develop a cross non-specific marker for resistance to the necrosis component of the tan spot disease of wheat, was not successful. Two separate attempts at bulk segregant analysis using different bulks from different cross populations were made. Failure in the first attempt could be attributed to the way in which the bulks were constructed and to the lack of genetic diversity between the resistant and susceptible parents of the crosses used. The origin of line 86ISWMN 2137 is not known, but it is suspected that it was developed in Eastern Canada. If this was the case, then close similarity between 2137 and the western Canadian cultivars Glenlea and Kenyon could be expected. However, in the second attempt

only one cross was used in formation of the bulks and the cross was between Red Chief, a resistant winter wheat, and Kenyon, a susceptible spring wheat. Thus the limitations of the first attempt had been removed, yet the results were as unproductive as in the first attempt.

This experience highlights limitations of the RAPD analysis. It is not useful or necessary reliable when working with polyploid species such as wheat which has a large genome containing many homeologous chromosomes and which appears to be highly conserved. Under these conditions, RAPD analysis reveals only a very low level of polymorphism and repeatability of results is poor, both among different laboratories and among crosses. In this study, RAPD markers which had been reported to be linked to resistance gene *tsn1*, failed to produce polymorphisms in the crosses used even though genetic studies indicated that gene *tsn1* was present in the resistant parents of those crosses.

Success in identifying useful RAPD markers also depends on the number of primers screened and the accuracy with which the component lines in each bulk can be phenotyped with respect to disease reaction. While the probability of success is directly related to the number of primers tested, the number of primers used was not felt to be an important factor contributing to lack of success since over 600 primers were tested in this study. Similarly, only lines showing high levels of resistance or susceptibility after repeated disease tests were chosen to form the bulks, so that the probability of incorrect phenotyping was considered very low. The resistant and susceptible disease phenotype for the necrosis component of this disease can be differentiated with minimal difficulty in growth room seedling tests.

Use of PCR primers developed from RFLP probe Xbcd, known to amplify the region adjoining the location of resistance gene *tsn1*, did reveal repeatable polymorphisms, but these were not of immediate value for tagging the resistance gene. Restriction digestion of the bands produced by these primers produced different fragment patterns, two of which appeared to be associated with different genomes. When these fragments were sequenced, a high degree of similarity was found indicating that the region surrounding the *tsn1* gene locus is highly conserved in both resistant and susceptible cultivars. This confirms the earlier suggestion that the lack of genetic diversity in the chromosome region being studied contributed to the insensitivity of the RAPD analysis. For polyploid species such as wheat, more powerful techniques such as AFLP or microsatellites would be more appropriate.

CONCLUSIONS

1. RAPD analysis was not successful in identifying markers for resistance to the necrosis component of the tan spot disease. Few polymorphisms were found and most were not repeatable.
2. PCR primers developed from a RFLP probe linked to resistance produced repeatable polymorphisms which after restriction digestion produced specific banding patterns that were associated with the A and the D genome of hexaploid wheat.
3. Sequencing of the bands produced by restriction digestion indicated that a high degree of similarity existed among them. Thus the region amplified by the PCR primers was highly conserved and was probably the reason for the failure to identify RAPD markers linked to the tan spot resistance gene.
4. The sequence data did identify certain polymorphic sites due to one or a few base pair differences and could possibly have been used to design markers. However, polymorphisms were not consistent across all resistant and susceptible parents indicating that such markers would be cross-specific and therefore of little value.

IDENTIFICATION OF MOLECULAR MARKERS FOR RESISTANCE TO SEPTORIA NODORUM BLOTCH

INTRODUCTION

Septoria nodorum blotch, caused by *Stagonospora nodorum*, is an important component of the leaf spot complex of durum and common wheat in western Canada. In Saskatchewan, *S. nodorum* is most common in the eastern and north-eastern regions and can attack both the foliage and heads of wheat plants. Tests conducted in these regions indicated that annual yield losses were in the range of 10-15% (G.R. Hughes, unpublished data).

Host resistance to septoria nodorum blotch is available and is the most cost-effective and desirable form of disease control. This resistance can be identified in seedling growth room tests and in the field. However, disease phenotyping is greatly affected by environmental conditions and consequently, accurate results cannot always be obtained without repeated testing. Molecular markers which could be used in selection for resistance, would eliminate this problem and speed up the resistance breeding process. Therefore, the objective of this study was to identify molecular markers which could be used in marker-assisted selection for resistance to septoria nodorum blotch in wheat.

MATERIALS and METHODS

Two random inbred line (RIL) populations were used in this study, a $F_{2:7}$ population of cross 86ISWMN 2137 x Kenyon and a F2:8 population of cross Red Chief x Kenyon. Lines 86ISWMN 2137 and Red Chief are resistant to septoria nodorum blotch. Lines were tested for resistance to septoria nodorum blotch in seedling growth room tests using the testing protocols described earlier.

Segregation data from these tests suggested that a single incompletely dominant gene controlled resistance in cultivar Red Chief whereas resistance in 86ISWMN 2137 was controlled by more than one gene, possibly up to three.

DNA Extraction

Leaf samples were collected from approximately100 lines of each population and stored at -70°C until DNA was extracted using, initially, the CTAB method described by Procunier et al. (1990). In later studies with population Red Chief x Kenyon, DNA was extracted with the Dneasy Plant Mini-Kit (Qiagen).

Bulked Segregant Analysis using AFLP

Two DNA bulks, each containing DNA from 10 lines, were created for the 86IWMN2137 x Kenyon population. The resistant bulk (SNBR) was based on resistant lines (rating 1) #'s 5, 22, 25, 31, 34, 46, 47, 50, 56 and 68; the susceptible bulk (SNBS) was based on susceptible lines (rating 5) #'s 1, 2, 9, 11, 13, 19, 20, 26, 64 and 65.

Initially, these bulks and the two parents were screened using RAPD with 200 random primers from the University of British Columbia. The protocol followed was very similar to that described earlier for the tan spot resistance marker study. Very few polymorphisms were detected and none were reproducible. Because of this lack of success, the AFLP (amplified fragment length polymorphism) procedure was used to try to identify markers. AFLP has been reported to be more powerful and reliable than RAPD in identifying potential markers when working with polyploid species such as common wheat, which contain much repetitive DNA.

AFLP Procedure

Because use of AFLP was new to our laboratory, time was needed to become familiar with the technique and to optimize it for our conditions. The procedure used followed the protocol of the Gibco-BRL AFLP kit. DNA is digested at 37°C for 2 hours using 2 µl Mse I/Eco RI and 5 µl 5X buffer in a final volume of 25 µl. The digested samples were heated at 70°C for 15 minutes and then chilled on ice.

Adapter ligation was achieved by combining 25 µl of the digested DNA, 24 µl of adapter ligation solution and 1 U of T4 DNA ligase and incubating the samples at 20°C for 2 hours. The

ligation mixture was diluted by 1:10 for use in pre-amplification. Pre-amplification was performed in 0.2 ml thin-walled reaction tubes containing 5.0 µl diluted template, 30 ng of E+1 and M+1 primers, 1x AFLP PCR buffer and 1 unit of Taq DNA polymerase. The PCR reaction was performed in a Perkin Elmer 9600 thermal cycler (Perkin Elmer Corp., Norwalk, CT, USA) using a temperature program profile of 20 cycles of 30 sec at 94°C, 60 sec at 56°C, and 60 sec at 72°C. The products of the pre-amplification reaction were diluted 1:50 for subsequent selective PCR amplifications.

For selective amplification of DNA fragments, 4 µl diluted DNA (1:50) was placed in a 0.5 ml thin walled reaction tube with 20 µl of a mixture including 30 ng of E- and M- primers which have the same sequences as the E+1 and M+1 primers but have 2 additional selective nucleotides at the 3' end of each primer, 1 U of Taq DNA polymerase and 1x AFLP PCR buffer. The PCR reaction used the temperature program of 1 cycle of 30 sec at 94°C, 30 sec at 65°C, 60 sec at 72°C, then 12 cycles using similar temperatures and time except that the annealing temperature was lowered by 0.7°C each cycle, and finally 23 cycles of 30 sec at 94°C, 30 sec at 56°C and 60 sec at 72°C. These PCR reactions were also performed in the PE-9600 thermocycler.

The PCR product was mixed with 20 µl loading buffer (98% formamide, 10 mM EDTA, 0.005% each of xylene cyanol FF and bromophenol blue), denatured at 90°C for 5 minutes then immediately placed on ice. 6% polyacrylamide gels (acrylamide-bisacrylamide 20:1) were prepared and pre-run at 85 watts for 1.5 hours in 1x TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA at pH 8.0). Then a 4 µl sample of each denatured PCR product was loaded per lane and the gel run for 3 hours at 80 watts.

After electrophoresis, the gel was fixed for 20 minutes with agitation in a 10% acetic acid solution, washed in ultra pure water for 3 times for 2 minutes each, and stained with a silver-stain solution (0.1% silver nitrate and 0.6% formaldehyde) for 30 minutes. The gel was then rinsed briefly in ultra pure water and quickly transferred to 2 L of developing solution (3% sodium carbonate, 3ml 37% formaldehyde and 400 µl sodium thiosulfate solution (10 mg/ml)). The gel was agitated well until the bands began to develop and was then transferred to fresh developing solution and developed for 2 to 3 minutes or until all bands were visible. Developing was terminated by adding 10% acetic acid and incubating the gel with agitation for another 2 to 3 minutes. Finally, the gel was rinsed twice in ultra pure water and dried for reading or photo-scanning.

Amplification of DNA samples of the Red Chief x Kenyon population was done in a Robocycler Gradient 40 thermocycler rather than the previously used PE-6000. Due to differences in

the thermocyclers, it was necessary to extend all steps in the PCR program by 30 seconds to get adequate amplification. No other changes were made to the AFLP protocol.

RESULTS AND DISCUSSION

Over the course of this study, three individuals were involved, Dr. Wenguang Cao (January, 1998 - July, 1998), Dr. Chunlin He (January, 1999 - November, 1999) and Ms. Carolee Erickson (March, 2000 - December, 2000). Dr. Cao did the RAPD analysis which was unsuccessful and resulted in the shift to use of AFLP analysis in which both Dr. He and Ms. Erickson were involved. Since the AFLP results obtained by each of them were not always the same, their results are presented separately.

Work done by Dr. Chunlin He (January, 1999 - November, 1999)

The parental lines and the SNBR and SNBS bulks of population 86ISWMN 2137 x Kenyon were screened with 68 primer combinations to detect polymorphisms. Eighteen primer combinations produced at least one visible polymorphism each (Table 1). Some of the primer combinations, such as M-CAG/E-ACC, M-CTA/E-AAG and M-CTG/E-AAG showed two polymorphic bands on the polyacrylamide gels.

Nine of the primer combinations produced repeatable polymorphisms and in five, the band was associated with resistance i.e. in coupling (Fig. 4). When these primer combinations were tested on 100 individual lines, it was found that seven were not linked to resistance. The remaining two primer combinations, M-CTC/E-ACG and M-CAA/E-ACG, were loosely linked with resistance at distances of 28 and 33 cM, respectively. However, this degree of linkage is not high enough for these markers to be useful in marker-assisted selection.

A major advantage reported for the AFLP technique is its ability to produce a large number of polymorphisms. Yet in the population used in this study, the frequency of polymorphism was low. The E+3/M+3 primer combinations produced less than 70 detectable polymorphisms, whereas others e.g. Lu et al. (1998), have reported more than 140 polymorphisms when different primer combinations were used, particular those with fewer selective nucleotides.

The reason for the low number is not known and could be due to a number of factors such as the polyploid nature of hexaploid wheat, the primer set used, inappropriate restriction enzymes used to generate the DNA fragments, a high degree of genetic similarity between the parents of the cross studied, or the confusing effect of more than one gene controlling resistance in this cross.

Table 1. AFLP primer combinations showing polymorphisms based on the banding patterns shown by the two bulks and the two parents

Primer combination	No. of polymorphisms	Coupling (+)/ Repulsion(-)	Repeatable[a]
M-CAA/E-ACA	1	+	Y
M-CAA/E-ACG	1	-	Y
M-CAC/E-AAG	1	+	Y
M-CAG/E-AAG	1		N
M-CAG/E-ACA	1		N
M-CAG/E-ACC	2	+ /+ [b]	Y
M-CAG/E-ACG	1		N
M-CAG/E-ACT	1		N
M-CAT/E-AAC	1		N
M-CAT/E-ACA	1	+	Y
M-CTA/E-AAG	2		N
M-CTA/E-ACG	1		N
M-CTC/E-AAG	1		N
M-CTC/E-ACC	1		N
M-CTC/E-ACG	1	+	Y
M-CTC/E-ACT	2	-/-	Y
M-CTC/E-AGG	1	-	Y
M-CTG/E-AAG	2	+/+	Y

[a] Y = repeatable and N = not repeatable.
[b] "+/+", "-/-" indicate both polymorphisms are in the coupling or repulsion phase, respectively.

Work done by Ms. Carolee Erickson (March, 2000 - December, 2000)

Work conducted during the first two months concentrated optimizing the AFLP protocol since the thermocycler being used was different to that used by Mr. Chunlin He who worked on this project previously. A protocol has been developed which appears to work on three different thermocyclers, which is valuable should the thermocycler normally used be unavailable or requiring repair.

The nine AFLP primer pairs that Dr. He had found produced repeatable polymorphisms were retested on the DNA bulks prepared by him from an F2:7 population of cross Kenyon (susceptible) x 2137 (resistant) and the parents. After testing all nine primer pairs, only seven were found to have reproducible polymorphisms. These tests were repeated and produced the same results. It is unknown if these polymorphisms are the same as those previously identified by Dr. He since he did not record the size of the previous fragments.

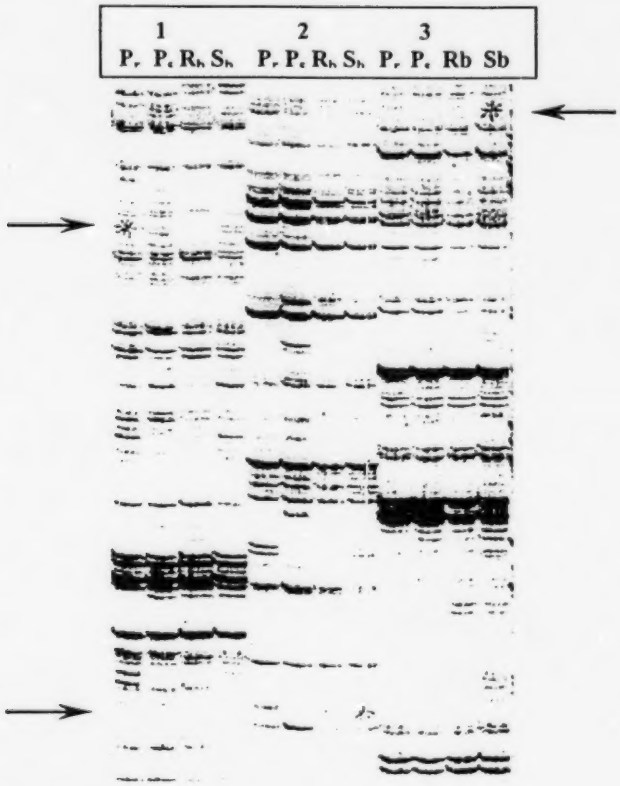

Fig. 4. AFLP banding pattern produced by three primer combinations, 1=M-CTC/E-ACT, 2= M-CAA/E-ACA, 3= M-CAC/E-AAG. Pr, Ps, Rb, Rs indicate the resistant and susceptible parents, and the resistant and susceptible bulks, respectively. Arrows indicate the positions of polymorphisms. Primer combination 1 shows a polymorphism in the repulsion phase (i.e. the band is associated with susceptibility), primer combinations 2 and 3 show polymorphisms in the coupling phase.

These seven primer pairs were then tested against the lines used to create the resistant and susceptible bulks. Two of them, M-CTC/E-ACG (polymorphism at ~ 190 bp) and M-CTC-/E-AGG (polymorphism at ~220 bp), showed segregation with resistance (both in repulsion). In both cases, only 3 of 18 lines tested did not segregate as expected. However, when these primers were tested on additional lines from the population, segregation was random and therefore these primers were discarded as marker possibilities.

Because of the lack of promising results with this cross, no further work with it will be done in the immediate future. Possible reasons for these poor results are that the parents are genetically similar, which reduces the possibility of finding polymorphisms and the disease reaction data suggested that resistance in 86ISWMN 2137 may be controlled by three genes. Both reasons complicate marker screening.

A population containing the winter wheat Red Chief as the source of resistance was chosen for further study. Genetic studies have shown that resistance in Red Chief is controlled by a single gene.

Preliminary Screening of Red Chief/Kenyon $F_{2:8}$ Lines

$F_{2:8}$ lines of the Red Chief x Kenyon population was evaluated for reaction to septoria nodorum blotch in growth chamber tests. The plants were scored on a scale from 1-5 (1 being resistant, and 5 being susceptible). DNA was extracted and bulked from six susceptible lines (rating 5) and from six resistant lines (ratings 1-2) to create the susceptible and resistant bulks, respectively. These bulks were tested with 112 primer combinations through selective amplification, and run on 6% polyacrylamide gels, visualized with silver stain. Of the 112 primer pairs tested, 72 pairs showed some polymorphism. Thirty of the best primer pairs were tested on DNA from the parental lines, Red Chief and Kenyon and 14 primers produced identical polymorphisms in both the parents and the bulks. At this point, the disease data was reviewed, and it was decided that new plants should be evaluated for disease reaction so that lines with a moderately resistant reaction (rating 2) in the resistant bulk could be replaced with lines with a high level of resistance (rating 1).

Re-evaluation and Secondary Screening

After additional F2:8 lines had been tested for disease reaction, DNA from 12 new lines was extracted and new bulks were prepared for AFLP; these bulks contained a few different lines from those in the previous bulks. The new bulks and the parental DNA were tested with 12 of the primers that produced polymorphisms when tested on the previous bulks. Repeatable polymorphisms were obtained for in four of these primer pairs. An additional 10 other primer pairs were also repeated with the new bulks. One pair produced polymorphisms with the bulks and parents.

DNA of the individual lines composing the bulks were prepared for AFLP and was tested with the first four primer combinations. Table 2 shows the scores and recombination resulting from the use of each primer pair with the 12 constituent bulk lines.

Because recombination using these primer pairs was low, all four primer combinations were considered to be potential markers. In order to test these primer combinations further, DNA of 12 more lines (6resistant, 6 susceptible) was extracted using the Qiagen DNeasy Mini-Kit. However, these newly extracted lines showed very erratic banding patterns (Figure 5). The banding pattern of each lane should be very similar, with the exception of the polymorphic region.

Table 2. Scores obtained using four different primer combinations with the 12 lines used to create the R (resistant) and S (susceptible) bulks. The number of recombinants obtained is listed in the last column (R/12) for each primer

		R						S						
	Bp	11	17	23	54	58	69	12	36	38	39	40	51	R/12
E-ACT/M-CTC	222	+	-	+	-	+	+	-	-	-	-	-	-	2
	185	-	-	-	+	-	-	+	+	+	+	+	+	1
E-ATT/M-CGC	126	-	-	-	-	-	-	+	+	+	+	+	-	1
E-ACG/M-CTG	350	+	+	+	-	+	+	-	-	-	-	-	-	1
E-ACT/M-CAT	222	+	-	+	-	+	+	+	-	-	-	-	-	3

After running a quality gel, it was discovered that the DNA quality was very poor, which probably caused incomplete restriction in the beginning of the AFLP process. Plant tissue was re-extracted from older plants in the greenhouse by both the CTAB DNA Extraction Method and the Qiagen DNeasy Mini-Kit, but poor quality DNA was still obtained. Therefore, 30 new lines were planted in the greenhouse for new DNA extraction. Since these lines were selected on the basis of their disease reaction in the previous generation, their disease phenotype will have to be verified by progeny testing them. This will be completed in the spring, 2001.

Figure 5. Erratic banding patterns produced by poor quality DNA used in AFLP.

DNA of 20 of the new lines was prepared for AFLP and tested with the four primers pairs (listed in Table 2) that had shown polymorphisms. Primer combination E-ACT/M-CTC produced polymorphisms at 222 bp, in coupling with resistance, and at 185 bp, in repulsion with resistance, in the resistant and susceptible bulks (Fig. 6). When tested on the 12 component lines of the bulks, only one recombination for the polymorphism at 185 bp was obtained, suggesting tight linkage (8 cM) between the marker and susceptibility.

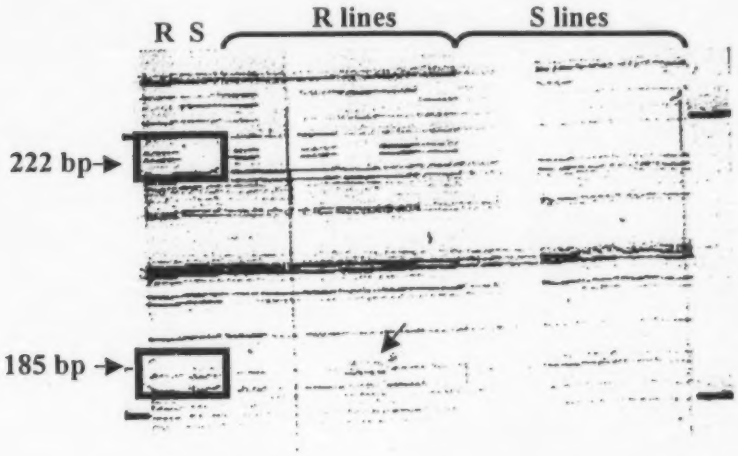

Figure 6. Banding pattern and polymorphisms (185 bp and 222bp) produced by primer pairs E-ACT/M-CTC when tested on the resistant (R) and susceptible (S) bulks and the 6 constituent lines of each bulk.

In a second analysis involving 20 individual lines, the parents and the bulks, this primer pair produced polymorphisms at 220 bp (linkage in coupling) and 180 bp (linkage in repulsion) (Figure 7).

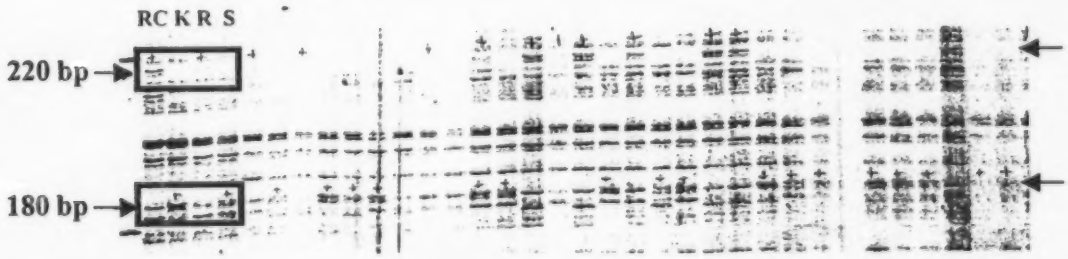

Figure 7. Polymorphisms produced by primer pairs E-ACT/M-CTC among the parents Red Chief (RC) and Kenyon (K) and the resistant (R) and susceptible (S) bulks, plus 20 individual lines.

Primer pair E-ATT/M-CGC produced a polymorphism at 126 bp when tested on the resistant and susceptible bulks; this polymorphism was linked in repulsion to resistance (Figure 8).

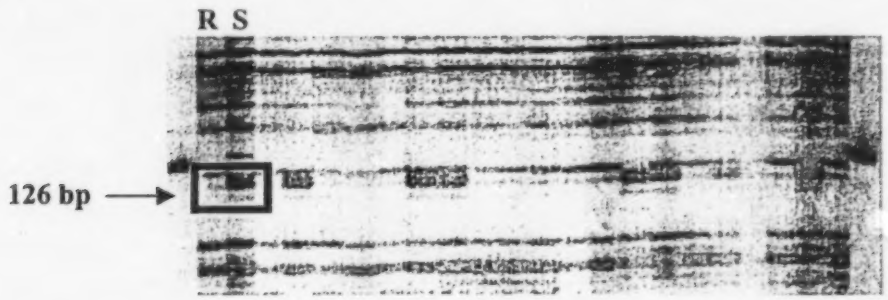

Figure 8. Polymorphism at 126 bp produced by primer pair E-ATT/M-CGC tested on the resistant (R) and susceptible (S) bulks and 20 additional individual lines.

Primer combination E-ACG/M-CTG produced polymorphisms at 350 bp (linkage in coupling) and 179 bp (linkage in repulsion) (Figure 9).

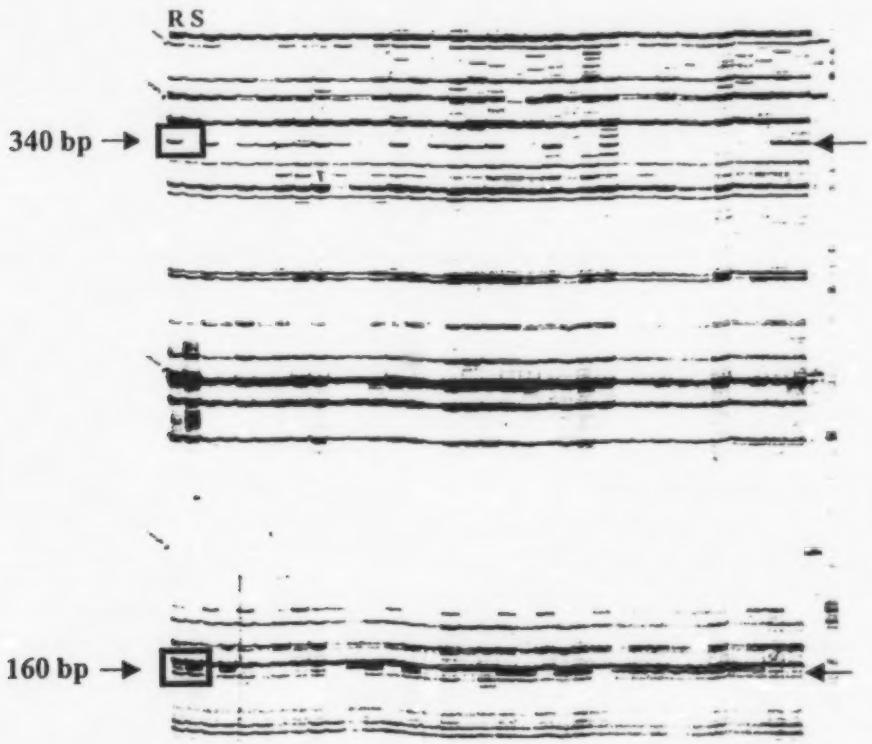

Figure 9. Two polymorphisms (160 bp and 340 bp) produced by primer pair E-ACG/M-CTG in the resistant (R) and susceptible (S) bulks (R and S) and the 20 additional individual lines.

Over the course of studying this second cross (Red Chief x Kenyon), four different sets of resistant and susceptible bulks were created and tested as newly extracted DNA and new disease scores became available. In total, 47 AFLP analyses were conducted to screen all of the possible primer combinations between 16 Gibco AFLP primers (E-primers, 8 M-primers) and 16 custom synthesized primers.

In addition to the five polymorphisms shown above (Figures 6 to 9), six other polymorphisms were identified that showed consistent association with either resistance or susceptibility when tested on the two parents, the bulks and the constituent lines of the bulks. In all cases very little recombination was found. Four polymorphisms were associated with resistance and seven with susceptibility. However, when each of the primer pairs that produced these 11 polymorphisms were tested on up to 37 individual lines from the Red Chief x Kenyon F2:8 population, most of the polymorphisms did not appear to be closely linked to resistance/susceptibility (Table 3).

The disease phenotype of each of the individual lines as determined in two different disease tests produced some conflicting results. Therefore, a "proposed phenotype" for each line was generated by averaging the scores of all 11 markers (Table 3). In most cases, this process verified the disease phenotype of the line, but in some lines the phenotype determined by the markers contradicted the disease data.

Thus lines could be separated into three groups: those where the proposed phenotype and the disease data agreed (Group 1); those where the proposed phenotype agreed with the disease reaction from one but not both disease tests (Group 2); and those where the proposed phenotype contradicted the disease data (Group 3). Data for lines falling into the last group was not considered when determining the degree of linkage between the markers and resistance/susceptibility. On this basis, linkage for the 11 markers tested was found to range from 12 cM (E-ACG/M-CTG 179 bp) to 43 cM (E-AAC/M-CGC 170 bp), with only five markers being linked at less than 20 cM (Table 3). When the data for only the Group 1 lines were considered, three polymorphisms (E-ACG/M-CTG 350 bp, E-ATT/M-CGC 126 bp, E-ACG/M-CTG 179 bp) were linked at distances of less than 10 cM (Table 4). Based on both estimates of linkage, the polymorphism produced at 179 bp by primer pair E-ACG/M-CTG appears to have the greatest potential as a useful marker. This marker is linked to susceptibility. Progeny testing of the individual lines is to be done in an attempt to establish the true disease phenotype of the individual lines. The final judgment of the value of these markers for marker-assisted selection for septoria nodorum blotch will be based on the disease data from these progeny tests.

Table 3. Polymorphisms produced by different AFLP primer combinations on the parents and individual lines from the cross Red Chief x Kenyon

Primer combination	Bp	Line no. RC[a]	Kyn[a]	13 1[b]	16 1	17 1	21 1	23 1	24 1	25 1	27 1	32 1	34 1	42 1	44 1	48 1	57 1	65 1	69 2	41 2
Polymorphism associated with resistance																				
E-ACT/M-CTC	222	+	-	+	-	-	-	+	+	-	+	+	-	?	?	?	?	?	?	+
E-ACG/M-CTG	350	+	-	+	+	+	+	+	+	+	+	-	+	?	?	?	?	?	?	-
E-ACA/M-CCC	126	+	-	-	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	-
E-AAC/M-CGC	170	+	-	+	+	+	+	+	+	-	-	+	+	-	-	-	+	-	-	+
Polymorphism associated with susceptibility																				
E-ACT/M-CTC	185	-	+	-	+	-	+	-	-	+	-	-	+	?	?	?	?	?	-	-
E-ATT/M-CGC	126	-	+	-	-	-	-	-	-	-	-	+	-	?	?	?	?	?	-	-
E-ACG/M-CTG	179	-	+	-	-	-	-	-	-	-	-	+	-	?	?	?	?	?	-	+
E-AGG/M-CGT	330	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+
E-AGG/M-CCG	100	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
E-ATC/M-CTA	135	-	+	?	+	-	+	-	-	+	-	-	+	-	-	+	+	-	-	-
E-ATA/M-CTC	350	-	+	+	+	-	+	-	+	-	-	-	-	+	-	+	+	-	-	+
Disease Phenotype (R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible)																				
Phenotype 1	Test 1	R	S	MR	MR	R	R	R	R	MR	MR	R	R	R	MR	R	R	R	R	R
Phenotype 2	Test 2	R	S	MR	MR	R	MR	MR	R	MR	MR	MR	R	MR	R	R	MR	R	R	MR
Proposed Phenotype		R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R?

[a] RC = Red Chief (resistant), Kyn = Kenyon)susceptible.

[b] Grouping of lines based on agreement between the disease phenotype indicated by actual disease tests and the predictive disease phenotype based on polymorphism produced. Group 1 = complete agreement, Group 2 = partial agreement, Group 3 = no agreement.

Table 3. (continued)

Line no.		30	11	18	19	20	22	33	36	38	39	51	12	37	40	14	15	28	29	54
Primer combination	Bp	2[b]	3	3	1	1	1	1	1	1	1	1	2	2	2	3	3	3	3	3
Polymorphism associated with resistance																				
E-ACT/M-CTC	222	+	+	+	-	+	+	-	-	-	-	-	-	?	-	-	-	-	-	-
E-ACG/M-CTG	350	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
E-ACA/M-CCC	126	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-
E-AAC/M-CGC	170	+	+	+	+	+	-	+	+	-	-	+	+	+	-	+	-	+	+	-
Polymorphism associated with susceptibility																				
E-ACT/M-CTC	185	-	-	-	-	+	-	+	+	+	+	+	+	?	+	+	+	+	+	+
E-ATT/M-CGC	126	-	-	-	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	-
E-ACG/M-CTG	179	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
E-AGG/M-CGT	330	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-	+	-	+	-
E-AGG/M-CCG	100	-	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+	-
E-ATC/M-CTA	135	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
E-ATA/M-CTC	350	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
Disease Phenotype (R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible)																				
Phenotype 1	Test 1	MR	MS	MS	S	S	S	MS	MS	S	S	MS	MR	MR	MR	MS	MS	MR	MR	MR
Phenotype 2	Test 2	MS	R	MS	MS	MS	S	S	S	S	S	S	S	MS	S	MS	MS	MS?	R	MR
Proposed Phenotype		R	R	R	S	S	S	S	S	S	S	S	S	S	S	S?	S?	S?	S	S

Table 4. Linkage of 11 polymorphisms with resistance or susceptibility to septoria nodorum blotch based on testing of individual lines from an Red Chief x Kenyon F2:8 population

Primer pair	Polymorphism	No. recombinants per		Linkage distance based on	
		All groups	Group 1 lines only[a]	All groups	Group 1 lines only
Polymorphism associated with resistance					
E-ACT/M-CTC	222	7/31	7/21	22.6 cM	33.3 cM
E-ACG/M-CTG	**350**	5/32	2/21	**14.6 cM**	**9.5 cM**
E-ACA/M-CCC	126	10/37	6/26	27.0 cM	23.1 cM
E-AAC/M-CGC	170	16/37	12/26	43.2 cM	46.2 cM
Polymorphism associated with susceptibility					
E-ACT/M-CTC	185	6/31	5/21	19.4 cM	23.8 cM
E-ATT/M-CGC	**126**	5/32	2/21	**14.6 cM**	**9.5 cM**
E-ACG/M-CTG	**179**	4/32	2/21	**12.5 cM**	**9.5 cM**
E-AGG/M-CGT	330	10/37	5/26	27.0 cM	19.2 cM
E-AGG/M-CCG	100	10/37	5/26	27.0 cM	19.2 cM
E-ATC/M-CTA	135	7/36	7/25	19.4 cM	28.0 cM
E-ATA/M-CTC	350	13/37	9/26	35.1 cM	34.6 cM

[a] Group 1 lines are lines whose disease phenotype agreed with the phenotype predicted by the average of the marker scores.

Although the primer combinations used in this study did not produce the large number of polymorphisms hoped for, the AFLP procedure was superior to RAPD analysis in the number of polymorphisms produced and appears superior when working with polyploid species, especially those with highly conserved genomes such as wheat. However, the increased number of polymorphisms produced did not translate into identification of many useful markers. Three primer pairs produced repeatable markers showing 12 to 15% recombination with resistance. When those lines whose disease phenotype was suspected to be inaccurate were removed, recombination for these putative markers decreased to 10%. This suggests that failure to identify additional useful markers may be the result of inaccurate disease rating of the lines studied, rather than difficulties associated with the AFLP protocol itself or the cross populations used. Progeny test data yet to be obtained may help resolve this question and identify which of the promising primer combinations would be useful for development of SCAR markers. This illustrates the "Catch 22" situation that can be encountered when seeking useful molecular markers. Markers are desired because of the difficulty of determining accurately the disease reaction of genotypes under test, but because of this difficulty, problems are encountered in determining accurately those markers which are useful.

CONCLUSIONS

1. The AFLP procedure was found to be superior to RAPD in identifying polymorphisms associated with resistance to septoria nodorum blotch in wheat.
2. The polymorphism produced by two primer combinations, E-ATT/M-CGC and E-ACG/M-CTG, are potentially useful markers. Both are linked in repulsion to resistance. However, further disease reaction data is needed to verify their linkage to the resistance locus and thereby establish their usefulness.
3. Mismatches between the disease test data and the disease phenotype suggested by the marker data contributed to difficulties in evaluating the value of polymorphisms identified. This emphasized the importance of accurate disease phenotyping through repeated testing and use of progeny tests.
4. Even though the AFLP procedure is powerful, it does appear to have limitations when working with polyploid species such as wheat where much of the genome is conserved. This is accentuated when the crosses studied possess restricted genetic variability.
5. Even more powerful techniques such as microsatellites should be examined for ability to identify markers in hexaploid wheat.

PERSONNEL

Dr. Wenguang Cao, Post-doctoral Fellow	0.4 PY
Dr. Chunlin He, Research Assistant	0.5 PY
Mr. Pawan Singh, Graduate Student	0.4 PY
Mr. Jerry Feng, Research Technician	0.1 PY
Ms. Carolee Erickson, Research Technician	0.8 PY
Dr. G.R. Hughes, Principal Investigator	0.3 PY

EQUIPMENT PURCHASED

None

ACKNOWLEDGEMENTS

The tan spot portion of this project formed part of Mr. Pawan Singh's Ph.D. thesis research and the support provided by this grant will be acknowledged in his thesis. Because of the negative results, I have no plans to publish this work further. The septoria nodorum blotch research will be published, and this project support acknowledged, if the linkage data for the potential markers is verified.

LITERATURE CITED

Faris, J.D., J.A. Anderson, L.J. Francl and J.G. Jordahl. 1996. Chromosomal location of a gene conditioning insensitivity in wheat to necrosis-inducing culture filture from *Pyrenophora tritici-repentis*. Phytopathology 86:459-463.

Lamari, L. and C.C. Bernier. 1989. Evaluation of wheat lines and cultivars for resistance to tan spot (*Pyrenophora tritici-repentis*) based on lesion type. Can. J. Plant Path. 11:49-56.

Lu, Z.X., B. Sosinski, G.L. Reighard, W.V. Baird and A.G. Abbott. 1998. Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks. Genome 41:199-207.

Procunier, J.D., X. Jie and K.J. Kasha. 1991. A rapid and reliable DNA extraction method for higher plants. Barley Genetics Newsletter 20:74-75.

Stock, W.S. 1996. Chromosomal location and RAPD marker development for tan spot resistance in hexaploid wheat. M.Sc. thesis, University of Manitoba, Winnipeg, Manitoba. 109 pp.